DEVELOPMENT AND CHARACTERIZATION OF CHITOSAN AND CHITOSAN-STARCH COMPOSITE SCAFFOLD PREPARED BY FREEZE GELATION METHOD

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CERTIFICATE

This is to certify that the thesis entitled

" DEVELOPMENT AND CHARACTERIZATION OF CHITOSAN AND CHITOSAN-STARCH COMPOSITE SCAFFOLDS PREPARED BY FREEZE GELATION METHOD*"*

Submitted by **Mr. Sonti Vamsy Krishna Sastry** in partial fulfilment of the requirements for the award of Bachelor of Technology Degree in Biomedical Engineering at National Institute of Technology, Rourkela is an authentic work carried out by him under my guidance.

To the best of my knowledge the matter embodied in the thesis has not been submitted to any University/Institute for the award of any Degree or Diploma.

Dr. Amit Biswas **Dated: 14TH MAY 2012**

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ABSTRACT

According to NIH "*Tissue Engineering can be defined as an emerging multidisciplinary field that applies the principles of biology, medicine, and engineering in order to improve the health and quality of millions of people worldwide by restoring, enhancing and maintaining tissue and organ functions*.one of its primary applications involves preparation of scaffolds both pure and composites for various tissue engineering applications. Natural polymers are one of the most looked into sources for preparation of these scaffolds, mainly because of their extracellular matrix [ECM] mimicking nature, unique chemical properties, and excellent biological performance. Chitosan is a deacetylated derivative of chitin, the most abundant biopolymer in nature and found in the walls of various fungi and shells of crustaceans. The proposed work is based on Freeze gelation method for the preparation of chitosan and its composite scaffold with the use of Starch. The effect of different weight ratios on the morphology, crystallinity, composition, biodegradability and biocompatibility is studied. This work will pave a way for economical scaffold manufacture for varied tissue engineering applications.

KEY WORDS: Starch, Chitosan, Composite, Freeze gelation.

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Chapter 1

INTRODUCTION

1.1 INTRODUCTION

Tissue engineering is an emerging interdisciplinary science which uses laboratory-grown tissues, artificial implants and materials to repair injured body parts and restore their functions. An ideal scaffold to be used for various tissue engineering applications should possess the following characteristics

- excellent biocompatibility,
- adequate pore size,
- controllable biodegradability,
- Suitable mechanical strength.

There is, therefore, an increasing need to look for new materials and methodologies to produce scaffolds for tissue engineering. One of the present trends in raw materials used for preparation of scaffolds is for materials that are derived from nature. Natural origin materials have been demonstrated to exhibit higher compatibility with human tissues and help in healing at a faster rate.

Natural polymers are one of the most looked into sources for preparation of these scaffolds, mainly because of their

- extracellular matrix [ECM] mimicking nature,
- unique chemical properties, and
- Excellent biological performance.

In this project Chitosan and starch are used as the requisite raw materials for scaffold preparation. Chitosan is a deacetylated derivative of chitin, the most abundant biopolymer in nature and found in the walls of various fungi and shells of crustaceans. Starch is a natural polymer that presents excellent characteristics for applications in the biomaterials field, primarily low toxicity biodegradability and biocompability. It is inexpensive and, above all, reusable.

Hence the proposed work is based on Freeze gelation method for the preparation of chitosan and its composite scaffold with the use of Starch.

The underlying principle involves thermally induced phase separation. The solution temperature is lowered to induce phase separation of the homogeneous biodegradable polymer solution. The process can either be liquid- liquid demixing which generates a

polymer rich and a polymer poor phase. The subsequent coalescence and growth of the polymer poor phase would develop to induce separation and formation of desired pores in the scaffold. But if we lower the temperature enough to allow freezing of the solution the separation would be solid- liquid demixing which forms frozen solvent and concentrated polymer phases. After removal of the solvent the remaining space becomes pores.

Composite materials can be defined as a material composed of two or more chemically and physically distinct phases (metallic, ceramic or polymeric), which are separated by an interface. In this process composites of chitosan and starch were prepared in varying ratios and were later characterized. The ratios were in order of 90:10, 80: 20, 70: 30, 60: 40 and 50: 50.

The samples prepared by using freeze gelation method were later characterized using various techniques outlined below.

Phase analysis was done by using X- Ray Diffraction and its results showed an increase in crystallinity in the composite sample than what was observed in the pure chitosan sample. The morphological studies were conducted using Scanning Electron Microscopy and the obtained images were analyzed for morphological changes. Elemental analysis was conducted by using EDS, FTIR and both the studies confirmed the presence of Chitosan and Starch. Finally degradation studies were conducted to observe the behavior of the prepared scaffolds in simulated environment and the findings were plotted in a bar chart.

This work ultimately will pave a way for economical scaffold manufacture for varied tissue engineering applications and help in establishing freeze gelation as the new scaffold manufacturing technique. At the same time studies done here will help in proving that composite Chitosan- Starch scaffolds are better than pure Chitosan ones.

Chapter 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

According to NIH "*Tissue Engineering can be defined as an emerging multidisciplinary field that applies the principles of biology, medicine, and engineering in order to improve the health and quality of millions of people worldwide by restoring, enhancing and maintaining tissue and organ functions*

In recent years Tissue engineering has also emerged as an excellent approach for the repair/regeneration of damaged tissue, with the potential to circumvent all the limitations of autologous and allogenic tissue repair[6, 7]. This technology aims at preparation of new tissues from cultured cells using isolation, purification and storage. The new tissue produced is plenty in number and robust in an in-vitro environment compared to whole tissue or organs[8, 9, 10.] This technology ultimately aims at the reduction of the number of people who suffer from tissue or organ faliures.

The therapies of damaged or lost tissues or organs include tissue or organ transplantation, surgical reconstruction, drug therapy, synthetic prostheses, and medical devices.

Tissue or organ transplantation is restricted by an insufficient number of donors. Although the other therapies are not limited by supply, they also have problems. For example, synthetic prostheses and medical devices are not able to replace all the functions of a damaged or lost organ or tissue. The efforts to address these problems and their limitations have elicited the development of new biomaterials and alternative therapies. Tissue engineering has emerged as one such promising alternative approach in treating patients suffering fromthese problems. Tissue engineering involves the expansion of cells from a small biopsy, followed by the culturing of the cells in temporary three-dimensional scaffolds to form the new organ or tissue. By using the patient's own cells, this approach has the advantages of autografts, but without the problems associated with adequate supply.

With this approach, porous three-dimensional temporary scaffolds play an important role in manipulating cell functions. Isolated and expanded cells adhere to the temporary scaffold in all three dimensions, proliferate, and secrete their own extracellular matrices. replacing the biodegrading scaffold. Therefore, in addition to permitting cell adhesion, promoting cell growth, and allowing retention of differentiated cell functions, the scaffold should be biocompatible, bioresorbable, bioerodable, highly porous with a large surface to volume ratio, mechanically strong, and capable of being formed into desired shapes.

Active seeding has some technical difficulties and obstacles to grow cells in sufficient quantities, urging their differentiation into the desired cell type, ensuring their blood and nutrient supply after implantation in the body.

The solution to this problem is the use of tissue engineering to "grow" organs in a controlled environment to replace diseased ones. In this relatively new and promising scientific field, the patients' own cells are grown from a small biopsy before culturing them on a temporary biodegradable polymer scaffold in a sterile environment. The tissue engineered organ is then transplanted into the patient without the slightest risk of tissue rejection of foreign cells by the body.In this innovative approach, polymer scaffold structures are extremely important.
Besides providing growth factors for cells and determining the shape of the eventual organ, these structures must have a highly porous structure with extensive interconnectedness and a high surface area so that cells have adequate space to grow in3. In addition, the polymer structure must not exhibit immunogenicity or cytotoxicity. Several tissue engineering methods have been carried out which fit these criteria. They include fiber bonding (unwoven meshes), solvent casting or particulate leaching, gas foaming, porogen leaching and phase separation or emulsification. In this research project, porogen leaching, solvent casting and particulate leaching were carried out.
In each method, several factors were varied to investigate the effect on the polymer scaffold formed. The bigger the pore size and pore surface area, the more suitable the method used to make the polymer because cells can grow in adequate space.

Potential strategies to replace repair and restore the function of the damaged tissues or organs include stem cell transplantation, transplantation of tissues engineered in the laboratory, and the induction of regeneration by the body's own cells[11-14]. Bone, cartilage, skin, cardiovascular prosthesis, and partial organ tissue regeneration and reconstruction are now possible and have shown promise for a large portion of individuals that have special needs because of tissue loss or organ failure[15]. To reconstruct a new tissue by tissue engineering, triad[16-18] components are needed. These include,

Scaffold: Growing cells in three-dimensional (3-D) matrices (scaffolds) or devices, where cells can be either recruited from the host tissues *in vivo* or seeded *in vitro*. Biomaterials are preferred as scaffold substrates due to their biodegradability, biocompatibility and non toxicity.

Cells: The use of isolated cells which are harvested from the donor tissue, (including nerve, liver, pancreas, cartilage, and bone as well as embryonic/adult stem or precursor cell) to replace those cells that supply the needed function, including genetic or other manipulations before the cell infusion.

Growth factors: Tissue-inducing substances, such as growth and differentiation factors promote and / or prevent cell adhesion, proliferation, migration, and differentiation by up-regulating or down-regulating the synthesis of protein, growth factors, and receptors.

Fabrication of tissue engineered scaffolds is an important research area in biomaterial engineering field.

2.1 Significance of scaffold

- Allow cell attachment and migration
- Deliver / retain cells and biochemical factors
- Enable diffusion of vital cell nutrients and expressed products
- Exert certain mechanical and biological influences to modify the behavior of the cell phase.

2.2 POLYMERS IN TISSUE ENGINEERING

They are a large class of natural and synthetic materials with a wide variety of properties. Polymers occur in nature and can be found in living species, such as proteins, collagen, and DNA. Synthetic polymers consist of a large group of materials that have become of common use in our life.

2.2.1 Natural polymers

Natural polymers can be classified as proteins (silk, collagen, gelatin, fibrinogen, elastin, keratin, actin and myosin), polysaccharides (cellulose, amylose, dextran, chitin and glycosaminoglycans) or polynucleotides (DNA, RNA). The macromolecular similarities of natural polymers with natural tissues generally increase biocompatibility and reduce immunologic responses. Scaffolds from natural polymers have been intensively studied in the past years. Collagen, Chitosan, Gelatin and Silk fibroin are some of the polymers studied for tissue engineering applications.

2.2.2 Synthetic polymers

Synthetic polymers have found increased applications as they are gifted with predictable and reproducible mechanical and physical properties such as tensile strength, elastic modulus and degradation rate. Typical biodegradable polymers used for biomedical purposes are hydrophobic polyester, such as polyglycolide (PGA) and polylactide (PLA), polyurethanes (PUs) and polyamides (PAs)

- *Hydroxyapatite (HA)*

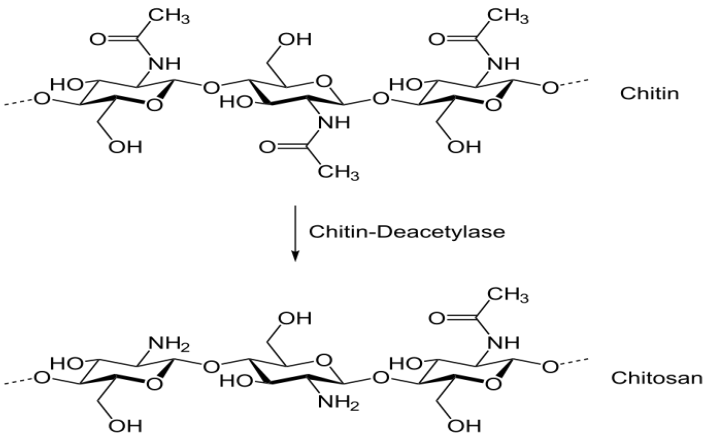
HA is a strong composite material similar to that found in actual bone apatite. Research has shown that when combined with low-moduli degradable polymers, HA acts to increase the modulus of the overall structure[1]. It is desirable to have the control to vary the modulus of a bioscaffold depending on the application.

- *Calcium Phosphate (CaP)*

CaP is much the same as HA. It is also a major component of bone that has been shown to increase scaffold modulus when combined with polymer materials[8]. Further, due to it being an actual component of bone it is highly biocompatible so no risks exist there. Once again modulus has been seen to increase with increasing amounts of CaP in polymer-CaP scaffolds meaning it would be possible to adjust the modulus of the scaffold for certain applications as mentioned for HA[8] .

- *Chitosan*

Ana Rita Costa-Pinto explained the use of Chitosan as a biomaterial for many potential applications. Chitosan is biocompatible, degradable, offer a wide range of properties and can be chemically modified to suit a wide range of biomedical applications and engineering. Chitosan can be used either alone or in combination with other biodegradable polymers, such as aliphatic polyesters, other natural polymers such as starch or silk, or with ceramics such as hydroxyapatite (HA) and TCP.



Source: Ana Rita Costa-Pinto

A number of different methods have been described for preparing porous structures to be employed as tissue engineering scaffolds. Each of these techniques presents its own advantages, but none are free of drawbacks. Different processing techniques have been developed for the design and fabrication of three-dimensional (3D) scaffolds suitable for TE implants. Fabrication of scaffold is done using natural and synthetic polymers by many conventional methods like electro-spinning phase-separation, solvent casting, particulate leaching, microsphere sintering, gas foaming, 3D-printing and CAD/CAM technologies[3].

- *Gas Foaming*

The gas foaming method involves taking solid discs of whatever material is being used and foaming them to create a porous structure. This works in the manner that the discs are placed into an airtight compartment where high pressure gas is slowly let in. The pressure is held very high for a number of hours. During this time the gas slowly creeps into the matrix of the material. After sufficient time has passed (varies for different materials) the pressure is released rapidly and the gas is forced quickly out of the matrix leaving porosity behind. The advantages of this method are that it is completed at low temperatures and no harmful solvents are used. However, through gas foaming the pore size and density cannot be easily controlled.[21]

- *Salt Leaching*

For the salt leaching method salt particles must be combined with the desired material for the scaffolds. By blending the materials evenly and melting them into solid discs they can then be placed into water for a few days until all the salt can be dissolved out of the material[3]. What this reveals is a porous structure. The advantages of this method are that it is completed at low temperatures and no harmful solvents are used. The other main advantage is the fact that pore size and density can be controlled based on the size of salt and the amount used.[4]

- *Gel Casting*

Gel casting begins first by dissolving the polymer in a solvent, which is then left at room temperature until a gel is formed. The gel is then extracted and processed through stages of solvent exchanges to yield a micro porous solid implant[2]. The advantages are that it can be completed at a low temperature and yield complex shapes[2]. However, the use of solvents to create the pores is not desirable, due to the fact that it is difficult to be able to remove all of the solvent from the scaffold after fabrication and if there is any solvent left in the pores of the scaffold it can lead to death of the cells that are trying to integrate into the scaffold[1].

Rapid Prototyping Techniques

The various Rapid prototyping techniques include 3DP and SLS. 3DP uses a water-based ink to print out rectangular bars of material, which is then particulate leached to create a porous structure. Selective Laser Sintering (SLS) builds up scaffolds one layer at a time as the powder is selectively bonded when a laser beam scans the powder[10]. Both of these methods allow for precise control over the microstructure of the scaffolds.

- *Electrospinning*

Electrospinning is a fabrication method for creating polymer nanofibers. The principle behind this methodology is that the material is melted and electrically charged by a high voltage source. This electrically charged polymer solution is then ejected out of a needle upon which it evaporates/solidifies before hitting a collection screen which collects the interconnected nanofibers[11]. These nanofibers are most useful for is reinforcing other composites by offering superior mechanical properties over microfibers made of the same material due to high surface: area ratio.

- *Freeze Gelation Process*

The underlying principle of freeze gelation involves thermally induced phase separation (TIPS). The solution temperature is lowered to induce phase separation of the homogeneous biodegradable polymer solution. The process can either be liquid-liquid de-mixing which generates a polymer rich and a polymer poor phase. The subsequent coalescence and growth of the polymer poor phase would develop to induce separation and formation of desired pores in the scaffold. But if we lower the temperature enough to allow freezing of the solution the separation would be solid-liquid de-mixing which forms frozen solvent and concentrated polymer phases. After removal of the solvent the remaining space becomes pores[5]

Nandhana studied the successful preparation of porous silk fibroin scaffolds by the freeze-gelation method. Silk fibroin was extracted from silk cocoons and the solution was kept for pre-freezing for 12hrs at -20 °c & -80ºc for 3-4 hrs. Frozen scaffold were immersed in 80% ethanol for 6-7 hrs at -20 °c. The scaffolds were washed with PBS and then dried at room temperature. The prepared scaffolds were highly porous, with porosity >90%, and have interconnected pores with pore size ranging from 60 to 110 µm. The prepared scaffolds are highly porous, with porosity >90%, and have interconnected pores with pore size ranging from 60 to 110 µm. HaCat cells cultured in scaffolds could attach spread and proliferate well on this porous freeze gelled scaffold confirming the applicability for cell culture studies[9].

Chien-Yang. investigated the influence of three important process variables (freezing temperature, concentration of acetic acid, and ethanol concentration) on the tensile properties of the chitosan scaffolds. Chitosan was dissolved in acetic acid and kept for freezing for 12hrs at -20 °c. The crosslinking agent was added to enhance the tensile strength of the scaffold. Frozen polymer solution was immersed in gelation environment for 12 hrs at -20 °c. The scaffold was washed with PBS and then dried in vacuum drier at 35 °c. Analyses of the process variables indicated that a higher freezing temperature and concentration of acetic acid in the scaffold solution increased the tensile stress and strain of the scaffolds at maximum load, while a high ethanol concentration in the rinse buffer only slightly increased the tensile stress of the scaffolds.[15]

Ming-Hua Ho. analyzed the preparation of porous PLLA, PLGA, chitosan and alginate scaffolds by freeze-extraction and freeze-gelation methods. The prepared scaffolds were highly porous, with porosity larger than 0.8, and had interconnected pores ranging from 60 to 150 µm. Compared with the freeze-drying method, the presented methods were more time

and energy efficient, with less residual solvent, and easier to be scaled up. It was observed that the ROS cells cultured in scaffolds could attach, spread, and proliferate well, indicating the potential applicability to tissue engineering of the scaffolds prepared by the proposed methods.[18]

2.3 COMPOSITES

2.3.1 Composite materials in tissue engineering

Composite materials can be defined as a material composed of two or more chemically and physically distinct phases (metallic, ceramic or polymeric), which are separated by an interface. Researchers have been applying composite materials in tissue engineering to enhance mechanical properties and cell function, and deliver special molecules. Polymers are known to be flexible and exhibit a lack of mechanical strength and stiffness, however they are simple to mould and can easily form complex structures, while ceramics are stiff. Composites aim to combine the properties of both materials to enhance tissue reconstruction.

Thein-Han described the synthesis of a bone-like organic–inorganic biomimetic nanocomposite consisting of chitosan and nHA for potential use as a bone tissue engineering material. Chitosan composite solution with 0, 0.5, 1 and 2 wt. % of nHA was prepared. The obtained pure chitosan solution or chitosan–nHA dispersion was transferred to polystyrene Petri dishes (area: 2 cm2), frozen at 20 ° C for 24 h and lyophilized in a freeze dryer. The pore size of the scaffolds were 55 to 115 µm and 45–100 µm, The study suggested that hydroxyapatite nanoparticles on the surface of chitosan–nHA nanocomposite scaffold significantly influence the morphology of attached cells.

Also, *Chang* reported the microstructure, mechanical performance, in-vitro bioactivity of chitosan-wollastonite composite scaffolds prepared by thermally induced phase separation method. Wollastonite powder in different ratios was added to chitosan solution and the mixture was frozen to solidify the solvent. The frozen scaffolds were then lyophilized. The wollastonite particles were uniformly dispersed on the pore walls of the scaffolds. Pore size was affected by freezing temperature; lower the temperature, better the pore size. An ideal scaffold to be used for bone tissue engineering should possess characteristics of excellent biocompatibility, adequate pore size, controllable biodegradability and suitable mechanical properties[1–3]. The choice of the appropriate fabrication technique is critical because it can significantly influence the properties of the implant and its degradation characteristics. There

is, therefore, an increasing need to look for new materials and methodologies to produce scaffolds for bone tissue engineering. One interesting possibility is to develop an in vivo responsive scaffold the properties of which may be regulated by the bone regeneration process, with gradual formation of pores in situ and consequent resorption. This hypothesis seems to be very promising due to the control of degradation in situ and the consequent pore formation, which allows the scaffold to have the required mechanical properties during the initial stage of implantation. One of the present trends in implantable applications is for materials that are derived from nature. Natural origin materials have been demonstrated to promote healing at a faster rate and are expected to exhibit greater compatibility with human tissues. The combination of chitosan with other materials appears to be a common theme in various reports[4,5].

The degradation of chitosan in the human body has been reported to be carried out by lysozyme. The degradation kinetics appears to be inversely related to the degree of deacetylation. Lysozyme, or muramidase, is an enzyme that catalyzes the hydrolysis of the peptidoglycan layer of bacterial cell walls. Human lysozyme is found in various body fluids in concentrations from 7 to 13 mg in serum and from 450 to 1230 mg in tears saliva and other fluids, including those surrounding cartilage. Following implantation of a biomaterial, neutrophils and monocyte-derived macrophages will be present around the foreign material in both the acute and chronic phases of inflammation. A number of enzymes, such as lysozyme, and reactive species will be released from these cells. Biodegradable starch-based polymeric biomaterials have been studied and proposed for a wide range of biomedical applications. Starch is one of the most abundant naturally occurring polymers, presenting a combination of properties that is steadily increasing its use in several technologies. Starch is a natural polymer that presents excellent characteristics for applications in the biomaterials field, primarily low toxicity, biodegradability and biocompability[20,21] . It is inexpensive and, above all, reusable. The main enzymes involved in starch degradation are a and b-amylase, glucosidase and other de-branching enzymes. Starch is hydrolyzed to glucose, maltose and dextrin. It is well known that salivary amylase is involved in the gastric and intestinal digestion of starch in food components. Amylase can also be found in human serum. The aim of this work was to develop a biodegradable matrix, based in chitosan and native starch that will form a porous structure in vivo by the preferential attack of the matrix by specific enzymes present in the human body (namely the a-amylase and lysozyme).

The inclusion of an enzymatically degradable phase in biomaterials may constitute an interesting approach to obtain scaffolds with adequate mechanical properties and with a gradual in situ pore forming ability. Using this innovative methodology, the developed scaffolds can exhibit very promising mechanical properties, due to the absence of macroporosity during the initial stage of implantation. The porosity is developed in situ by enzymes present in human body. In this work, chitosan/starch scaffolds were developed using a precipitation method. These systems were analyzed in terms of morphology, degradation behavior and mechanical properties. This study also addressed the effect of leachable from developed scaffolds on the viability of mouse fibroblasts and the influence of the construct's surface on the morphology, adhesion and spreading of fibroblast and human osteoblasts development of a biodegradable matrix, based on chitosan and starch, with the ability to form a porous structure in situ due to the attack by specific enzymes present in the human body (a-amylase and lysozyme). Scaffolds with three different compositions were developed: chitosan (C100) and chitosan/starch (CS80-20, CS60-40). Compressive test results showed that these materials exhibit very promising mechanical properties, namely a high modulus in both the dry and wet states. The compressive modulus in the dry state for C100 was 580 ± 33 MPa, CS80-20 (402 ± 62 MPa) and CS60-40 (337 ± 78 MPa). Degradation studies were performed using a-amylase and/or lysozyme at concentrations similar to those found in human serum, at 37 C for up to 90 days. Scanning electron micrographs showed that enzymatic degradation caused a porous structure to be formed, indicating the potential of this methodology to obtain in situ forming scaffolds.

Chapter 3

EXPERIMENTAL

3.1 WORK PLAN

1. Development of porous Chitosan scaffold by Freeze Gelation method

1. Preparation of 1M acetic acid solution with by mixing 5.6 ml of glacial acetic acid in 94.5 ml of distilled water;
2. 2.5 gm of Chitosan was dissolved in the above prepared solution and kept for stirring for 24 hours. The stirring was continued until a complete homogeneous polymer solution was obtained.
3. Pour the polymer solution in petri dishes and keep it in the deep freezer at -20°C for 24 hours.
4. Prepare the gelation media with 3M NaOH/EtOH by mixing 12 gm of NaOH in 95 ml of distilled water and then 1 M ethanol was added to mixture in 70: 30 ratios. The formed solution was kept for freezing at -20°c.
5. Immerse the frozen polymer solution in the gelation media and keep it in the freezer again for 24 hrs.
6. Remove the petridishes from freezer and the sample was dried at 45°C for 12 hours until complete evaporation of the solvent occurred.
7. Wash the scaffold with distilled water and ethanol to remove the traces of acid.
8. 1.5gm of Starch powder was added to 100 ml of distilled water and kept for stirring for 12 hours. The prepared starch solution was added to the prepared Chitosan polymer solution in varying quantities for making scaffolds of different ratios. The mixture was stirred over night at room temperature. Then the above mentioned protocol was followed.

2. Characterization of scaffold

- **Scaffold material characterization**
  - FT-IR and XRD
- **Microstructure analysis**
  - SEM, and EDS analysis
- **Degradation**

3.2 METHODS AND MATERIALS

Materials

- Chitosan (degree of acetylation >90 degrees) (obtained from SIGMA Chemicals India)
- Starch (Obtained from SIGMA Chemicals India.)
- Glacial Acetic Acid (99%) (purchased from Merck chemical laboratories)
- Sodium Hydroxide (NaOH) from Merck Chemicals.
- Ethanol is also obtained from Merck Chemicals.

Preparation of chitosan scaffold:

Chitosan was dissolved in acetic-acid aqueous solution to form a 2.5% polymer solution. The polymer solution was placed in a Petridish and frozen at -20°C for 24 hrs. The frozen Chitosan solution was immersed in a NaOH aqueous solution to adjust its pH to allow for the gelation of Chitosan. The NaOH aqueous solution was pre cooled so that the gelation occurred below the freezing point of the chitosan solution. The scaffold is kept in the vacuum drier overnight. The scaffold is cut into exact dimensions and made ready for different characterization techniques.

Table 1 showing the various solutions prepared for the process of scaffold preparation

Sample .No.	Chitosan (%)	Starch (%)
1	100	0
2	90	10
3	80	20
4	70	30
5	60	40
6	50	50

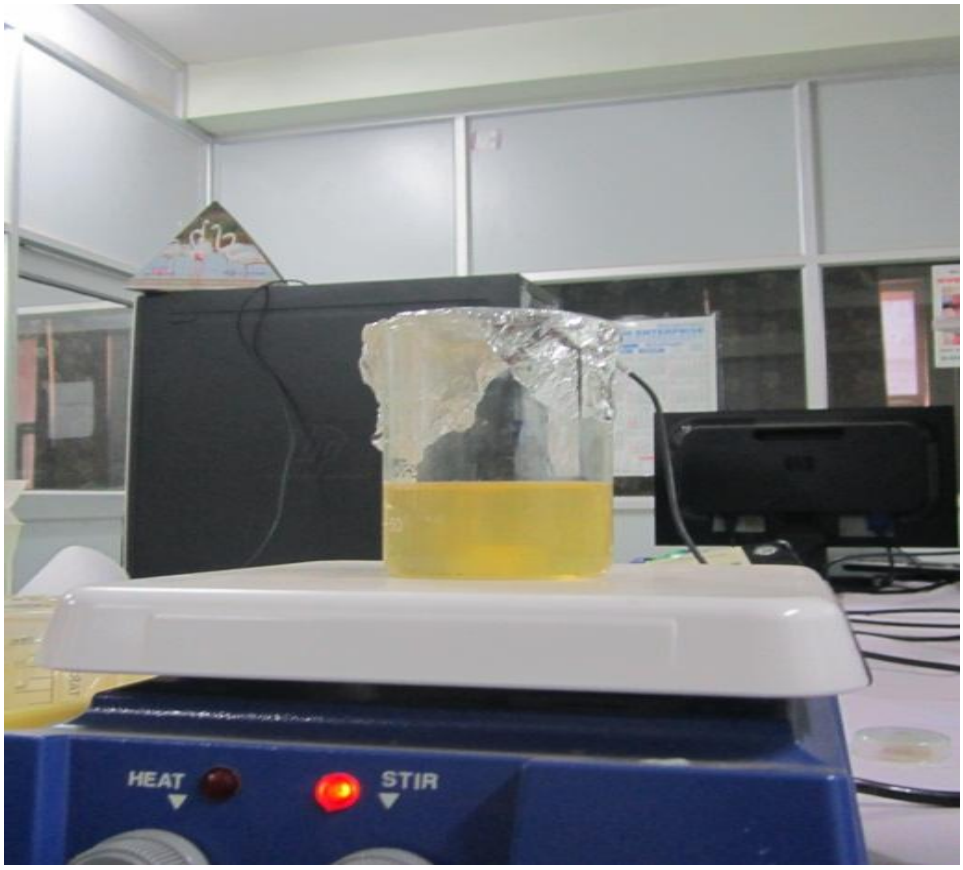


Fig 3.2.1 Preparation of Chitosan polymer solution

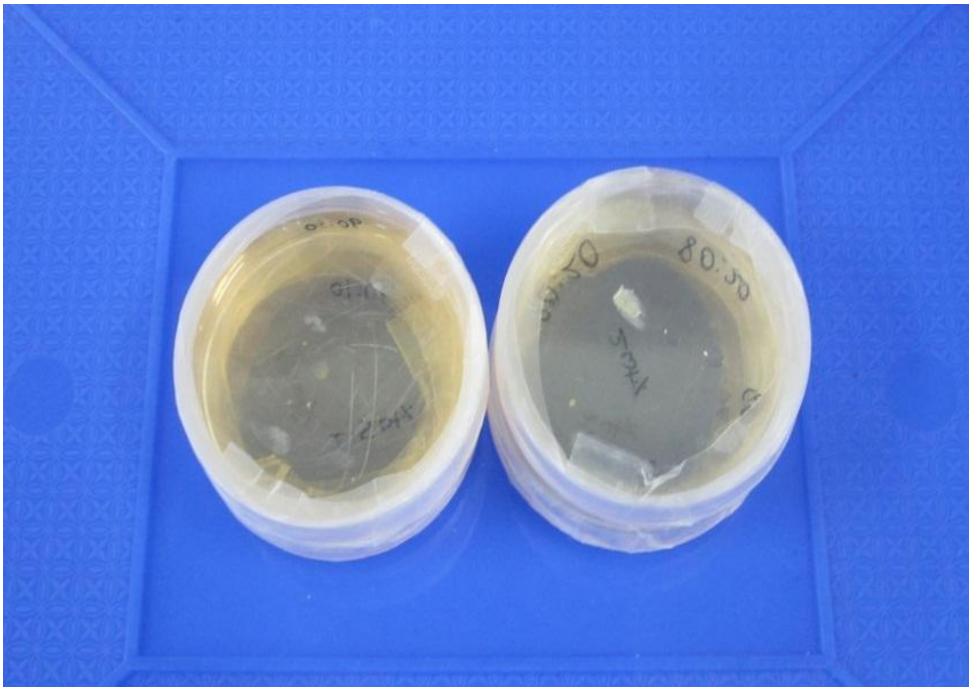


Fig 3.2.2 Polymer solution is poured in the petridishes and sealed with parafilm

Preparation Of Chitosan/ Starch Composite Scaffold

Necessary amount of Starch powder was added in order to prepare solutions of the desired ratios.

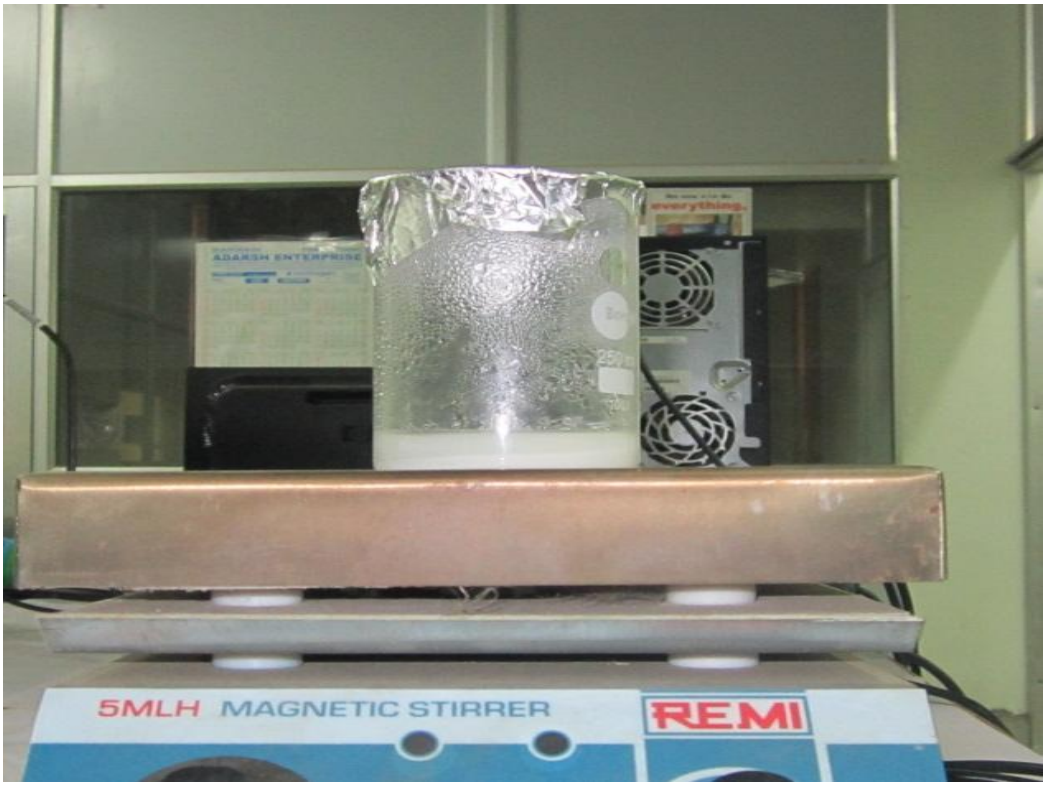


Fig 3.2.3 Preparation of starch solution

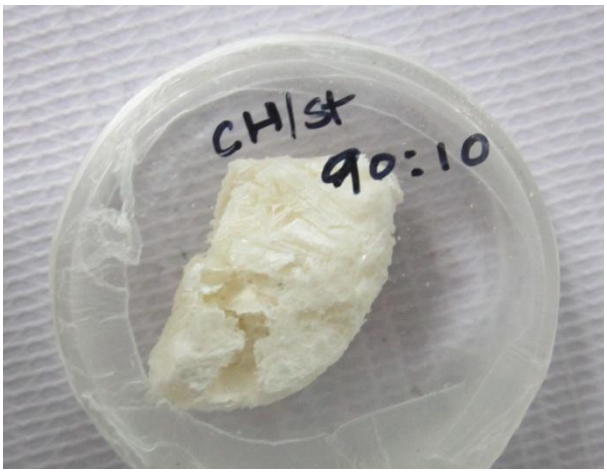


Fig 3.2.4. Chitosan Starch composite scaffold in the Wt. ratio of 90:10

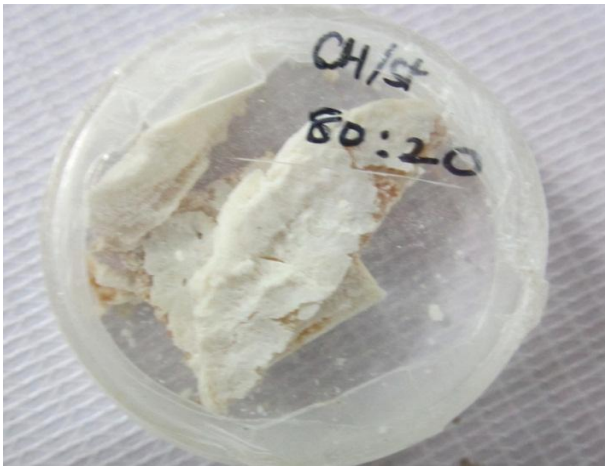


Fig 3.2.5 Chitosan Starch composite scaffold in the Wt. ratio of 80:20

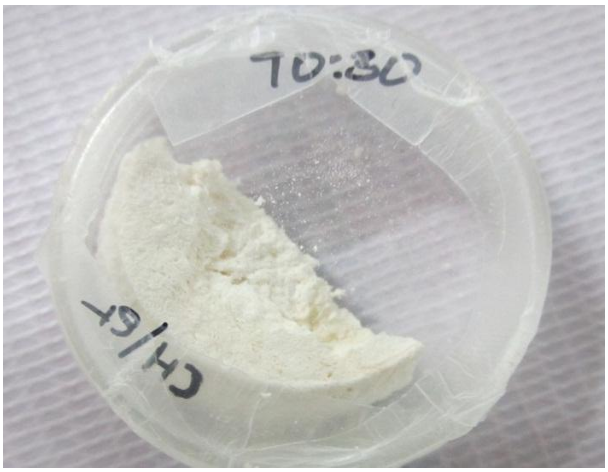


Fig 3.2.6 Chitosan Starch composite scaffold in the Wt. ratio of 70:30

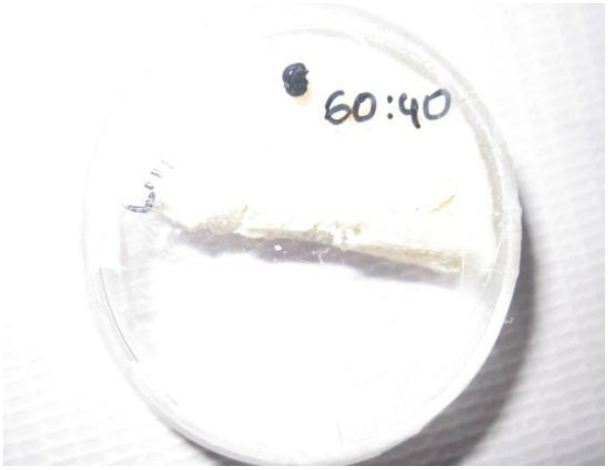


Fig 3.2.7 Chitosan Starch composite scaffold in the Wt. ratio of 60:40

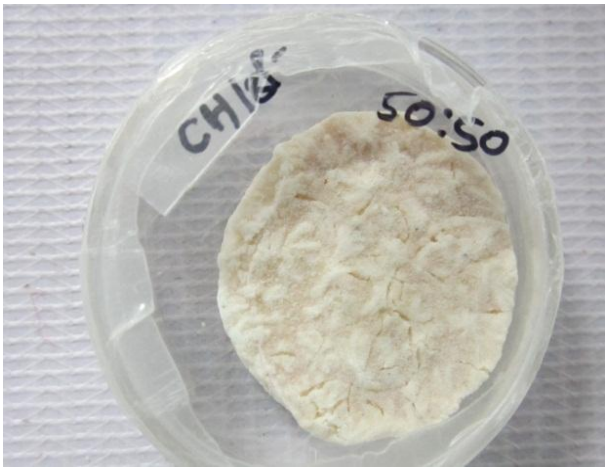


Fig 3.2.8 Chitosan Starch composite scaffold in the Wt. ratio of 50:50

3.3 CHARACTERIZATION

Scanning electron microscopy (SEM) was performed using a JEOL-JSM 6480 LV SEM to observe the morphologies of CS, Starch/CS composite scaffolds. The pore size of pure and composite scaffold was calculated by using the ImageJ software. The phase analysis of Chitosan and Chitosan-Starch composite scaffolds were analyzed by X-ray Diffractometer (XRD, D/max 2550 VB + /PC, X'pert Philips) using Cu Kα radiation in a step-scan mode in the 2θ range of 10-50°. Fourier transform infrared spectroscopy was performed in AIM-800 Automatic Infra-red Microscope (SHIMADZU) was used to determine the structural information of the CS and Starch/CS composite scaffolds.

Chapter 4

RESULTS AND DISCUSSIONS

4.1 Results and discussions

The scaffolds obtained by the freeze gelation process are as porous as the scaffolds obtained by the original freeze extraction technique. But the process of freeze gelation is faster easier consumes less time and money and produces scaffolds without the problem of skin formation.

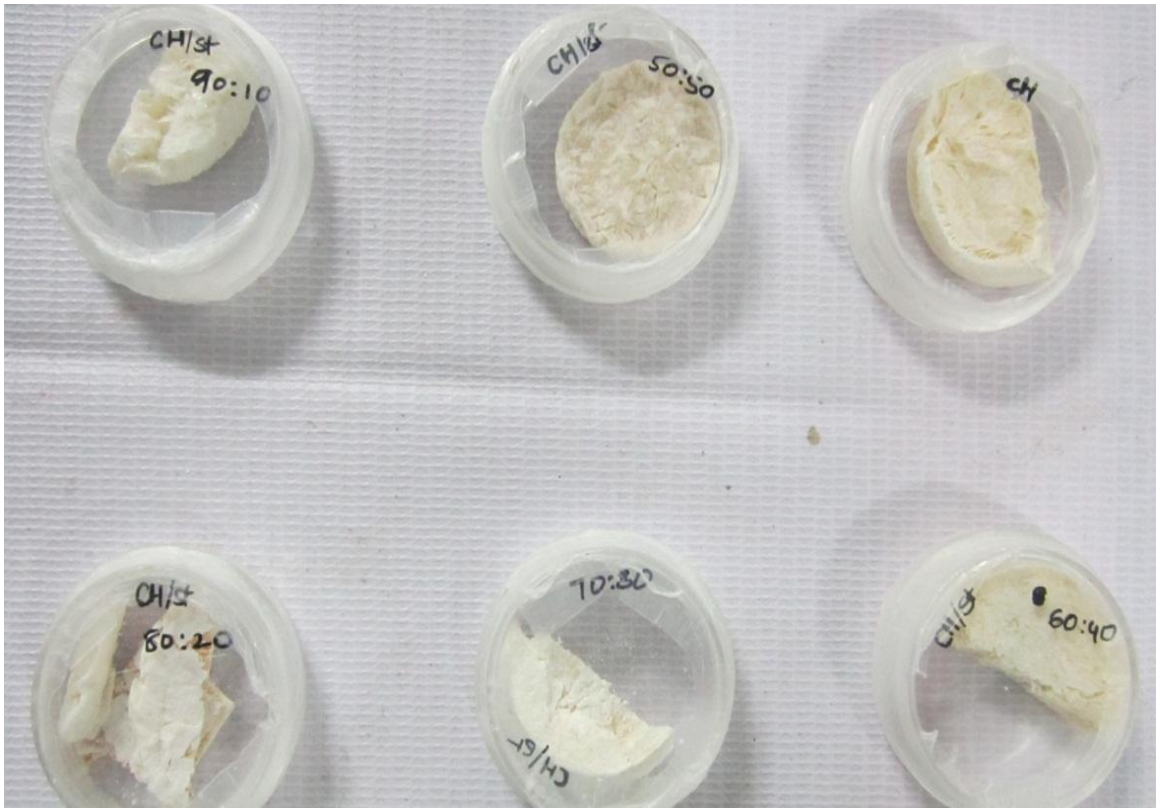


Fig 4.1.1 Prepared Chitosan and composite scaffold

4.2 Morphological Characterization

The microstructures, such as pore size, pore distribution, and pore morphology of the scaffolds were observed with a scanning electron microscope (SEM). SEM results show that both pure Chitosan and Chitosan/Starch composite have interconnectivity of pores. But the average number of pores nearly remains same in pure Chitosan as well as the composite one. The pore size of pure and composite scaffold was calculated by using the Image j software.

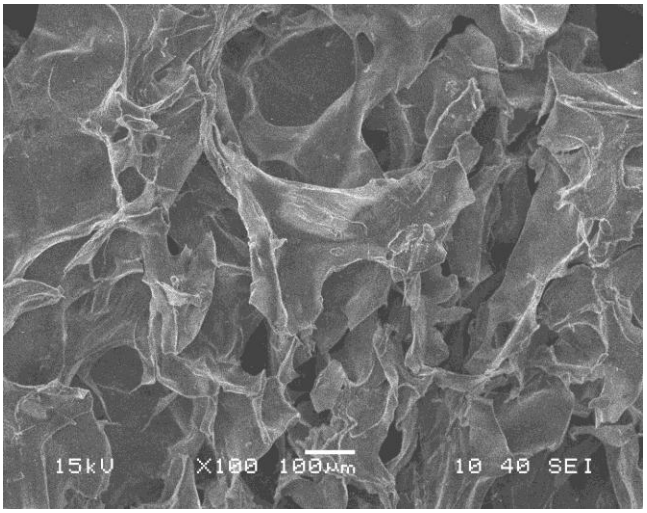


Fig.4.2.1.SEM image of pure Chitosan freeze gelled scaffold

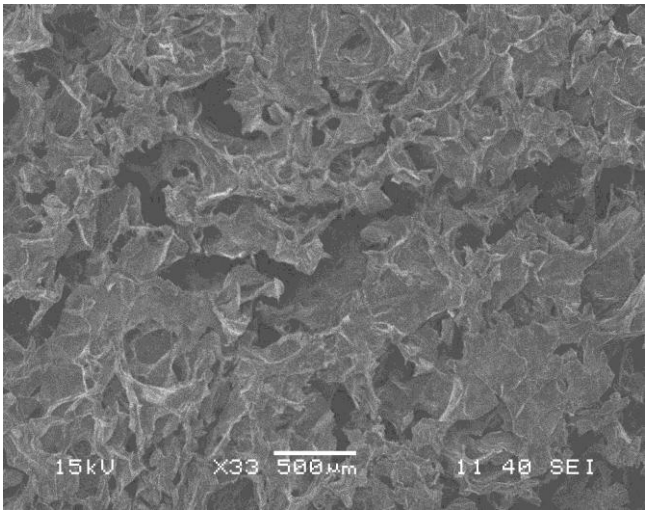


Fig.4.2.2.SEM image of pure Chitosan freeze gelled scaffold

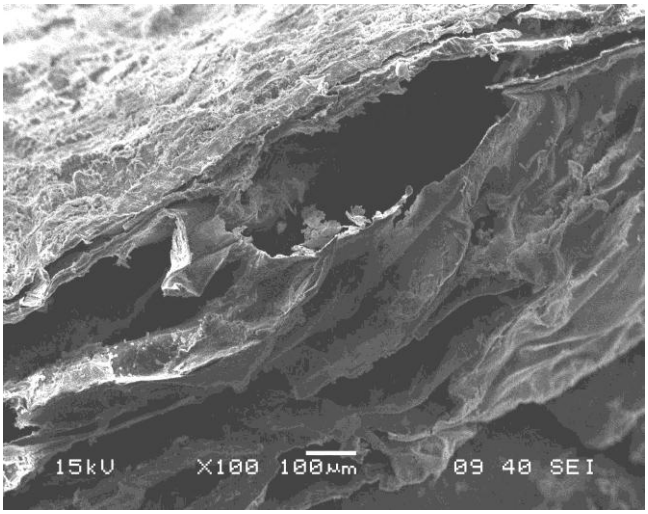


Fig.4.2.3 SEM image of Chitosan-Starch freeze gelled composite scaffold (90:10)

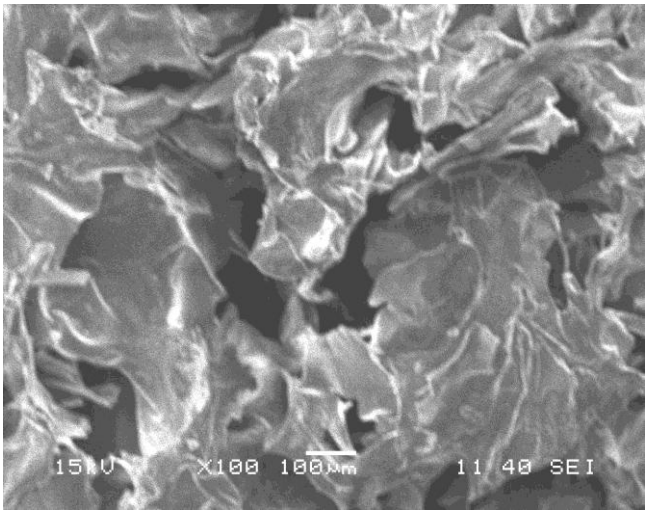


Fig.4.2.4 SEM image of Chitosan-Starch freeze gelled scaffold (80:20)

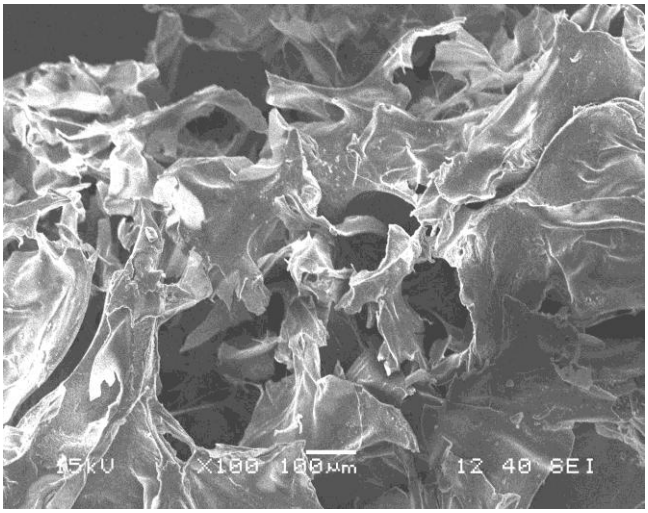


Fig.4.2.5 SEM image of Chitosan-Starch freeze gelled scaffold (70:30)

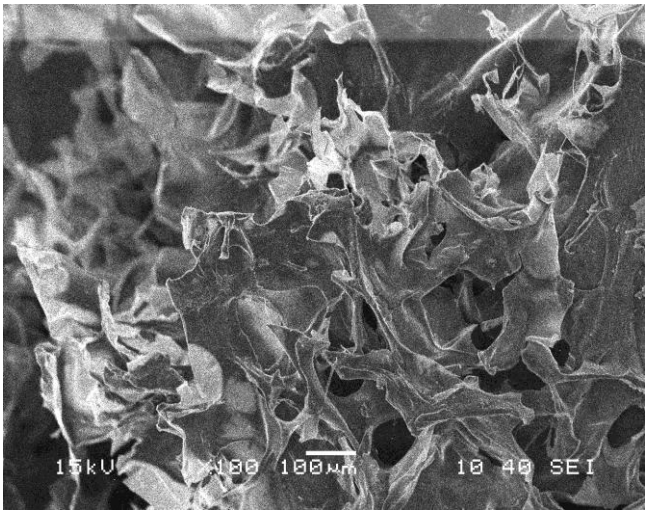


Fig.4.2.6 SEM image of Chitosan-Starch freeze gelled scaffold (60:40)

Table 4.2.7: Pore sizes of different chitosan scaffolds by freeze gelation

S.NO	SAMPLE	Pore SIZE (µm)
1	Plain CS scaffold	231.98±8
2	90 : 10	194.19±10
3	80 : 20	173.36±9
4	70 : 30	172.23±10
5	60 : 40	165.56±20
6	50 : 50	150.48±10

The obtained pore sizes vary from 150-230 µm**.** From the above images and analysis it can be clearly observed that the best outlined pores with the optimum pore distribution is obtained in the (70 :30) ratio sample of prepared Chitosan-Starch scaffold which is of 172.23±18 µm . The morphological analysis of the obtained images yields the results that the intermixing of starch in the Chitosan matrix is observed in all the composite scaffolds. Also the formation of surface skin (flaky skin like structures) is also observed in all of the samples. However the best pore formation and distribution is observed in the 70: 30 compositions.

4.3 Phase Analysis

The pure chitosan sample is semi crystalline in nature with a nimbus that peaks around 2θ of 20°. The reflection at 20° corresponds to the regular crystal lattice of chitosan.

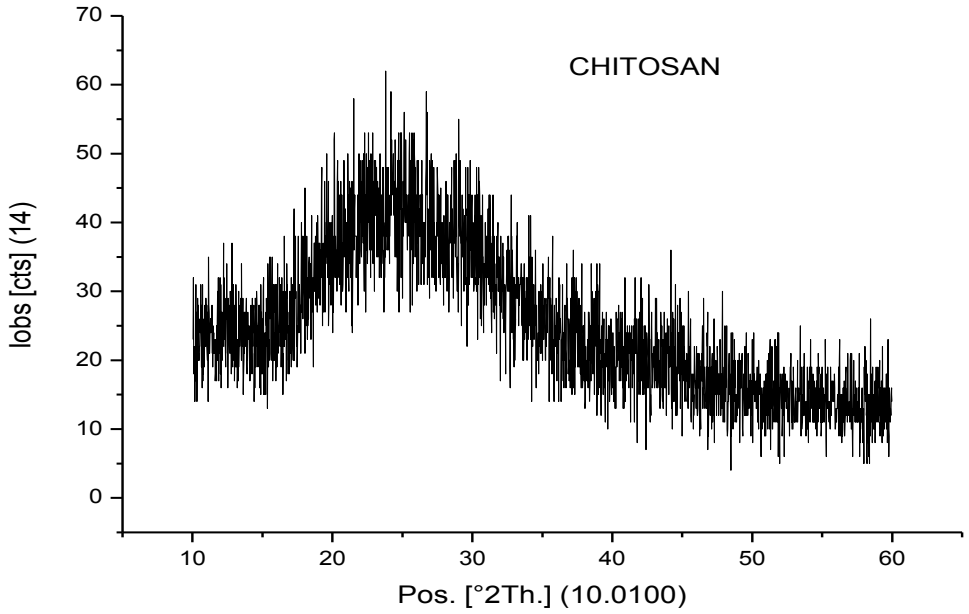


Fig.4.3.1 .XRD Plot of pure chitosan scaffold

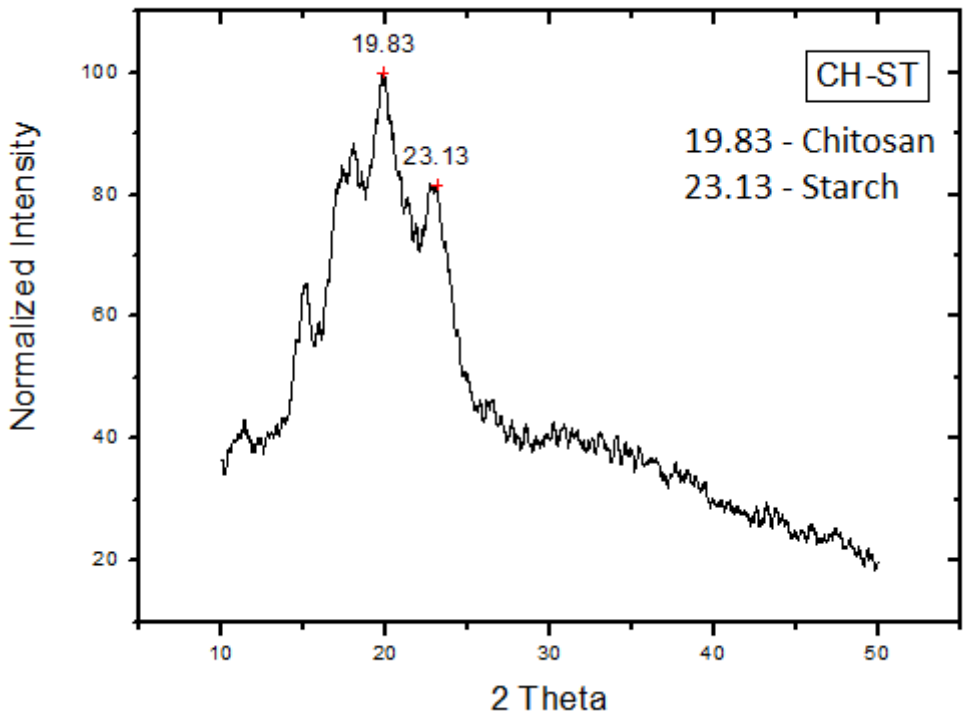


Fig.4.3.2. XRD graph of Chitosan-Starch composite scaffold (70: 30) ratio.

In the composite scaffold because of the presence of Starch the semi crystalline form is turned to more crystalline and the peaks are clearly visible at 2θ =20° to 50° with the highest intensity of about 300 counts at 2θ =22°,which is shown in fig.2.

From these diffractograms, it is obvious that composite scaffold is more crystalline than pure chitosan scaffold.

4.4 Elemental Analysis

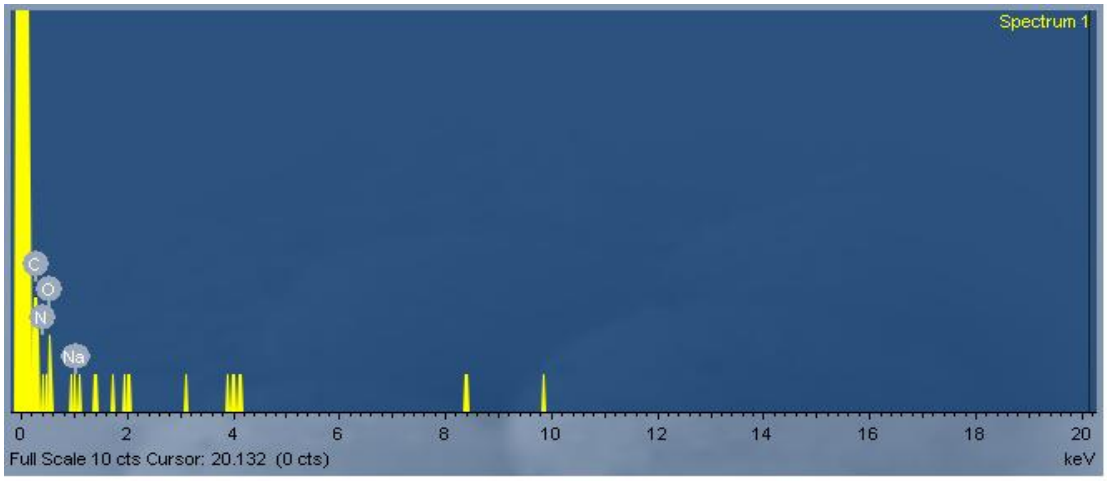


Fig.4.4.1 .EDS Spectra of chitosan freeze gelled composite scaffold

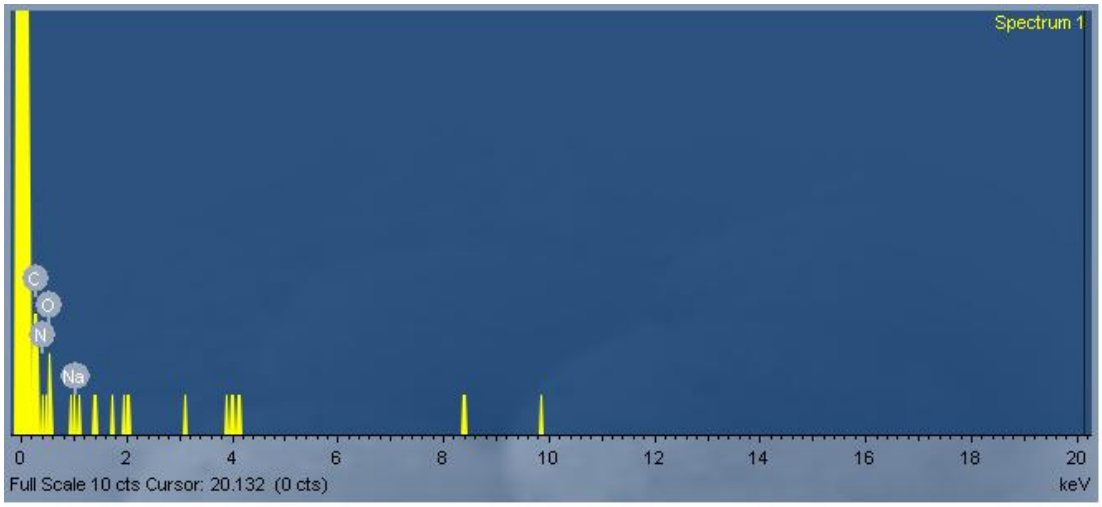


Fig.4.4.2 .EDS Spectra of chitosan freeze gelled scaffold (70: 30 ratio)

The EDS study clearly highlights the presence of elements Carbon, Hydrogen, Oxygen Sodium and Nitrogen. The elements carbon hydrogen and oxygen are present both in Chitosan and Starch. However Nitrogen is obtained from the amide groups present in Chitosan and Sodium is obtained from the Gelation medium.

4.5 Study of Infrared Spectrum

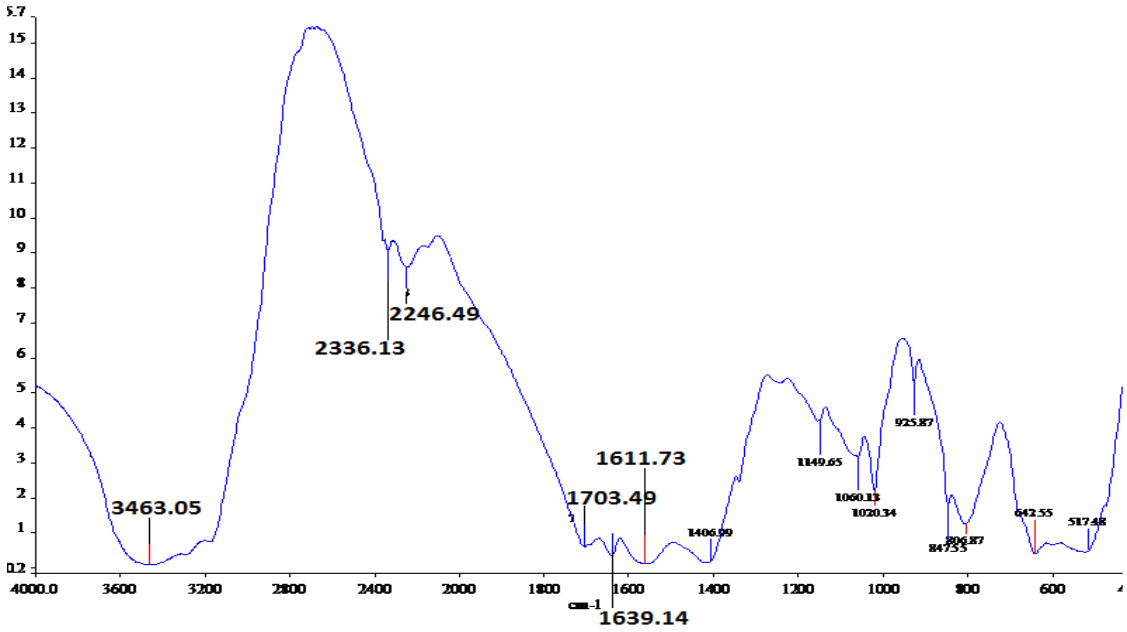


Fig.4.5.1 .FT-IR of pure Chitosan scaffold

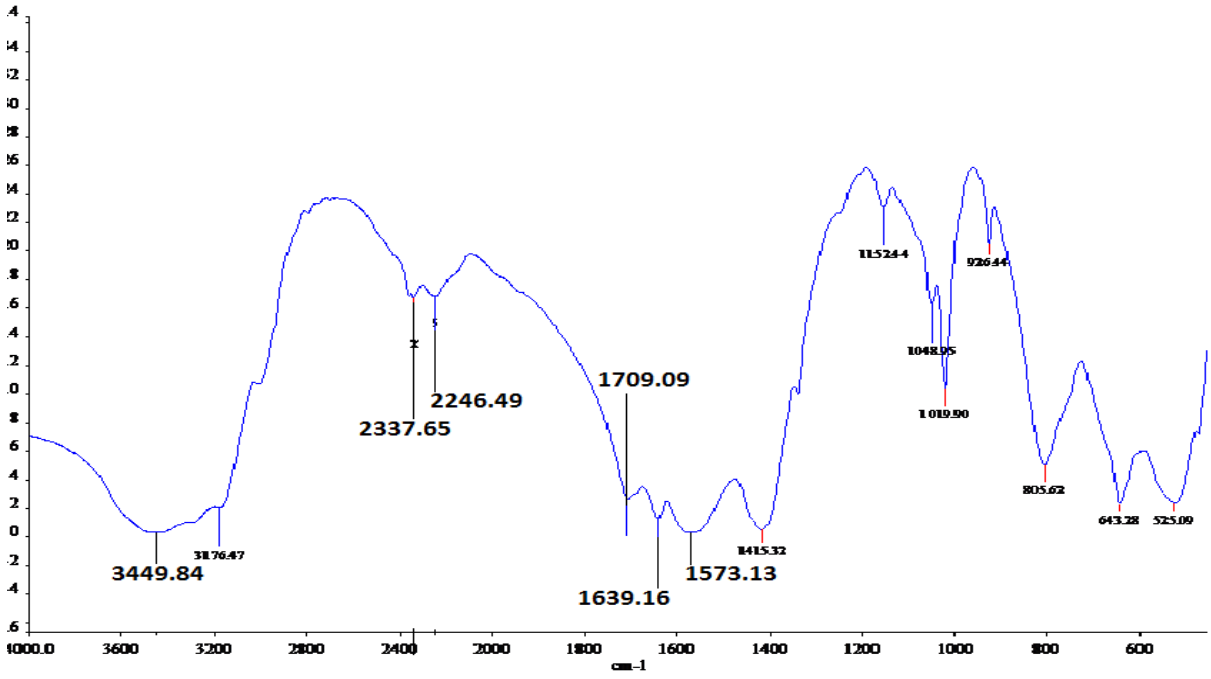


Fig.4.5.2 .FT-IR of Chitosan Starch composite scaffold (70: 30)

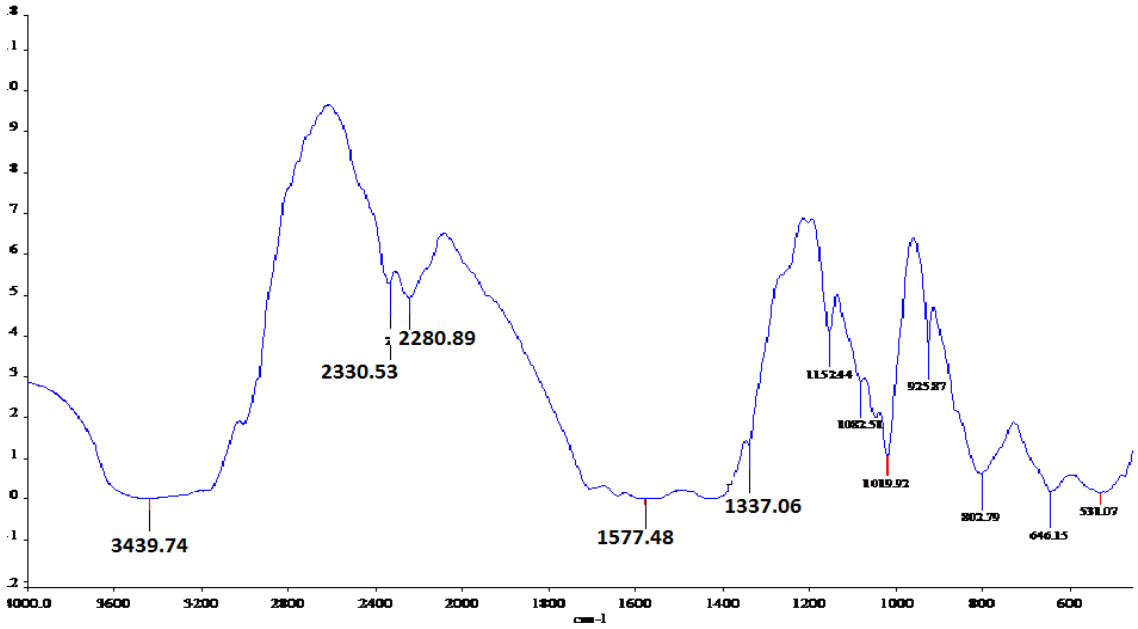


Fig.4.5.3 .FT-IR of Chitosan Starch composite scaffold (60:40)

The following study was carried out using the principle of Fourier Transform Infrared Spectroscopy. The standard curve for chitosan showed the following absorption peaks. The absorbance at around 1500 cm^{-1} is due to N-H bending. The peak at 3463 indicates presence of alcoholic group could be because of ethanol used in the gelation medium. The 3 peaks between 1570-1687 cm^{-1} shows the scissoring mode of N-H bond. The absorbance of 2240-2260 cm^{-1} is due to C-N stretching. All the FTIR images clearly verify the bonds pertaining the presence of amide group, alcohol (ethanol), carboxylic acids and multiple number of bonds between carbon hydrogen oxygen etc. however due to addition of starch into chitosan for formation of composites shifting of peaks has occurred but no startling differences exist between the images obtained from the various composites.

4.6 Degradation study

In degradation studies a small portion of the initial prepared sample of both pure Chitosan and Chitosan-Starch scaffolds was taken and its weight was noted down. Next it was immersed in PBS solution for 24 hours. After the stipulated time period the sample was taken out cleaned with tissue paper and again weighed. The change in weight was noted down. The PBS solution was changed and the same sample was reimmersed for another 24 hours. The process was repeated for the required number of days. Finally a bar chart was prepared highlighting the loss of weight of the sample after every 24 hours.

The formula used is $\mathbf{W_{lost} = (W_{initial} - W_{final} / W_{initial})*100\ \%}$

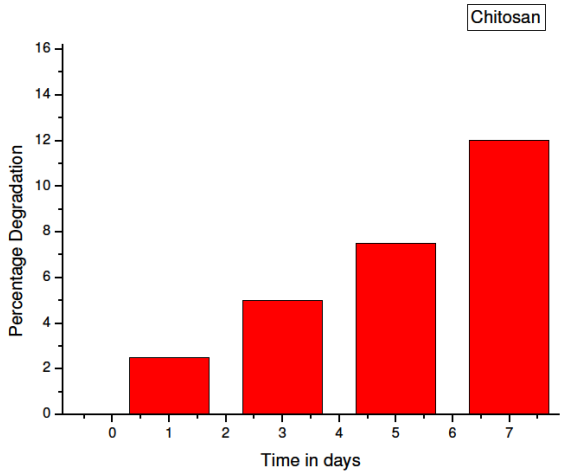


Fig.4.6.1 .Degradation study of pure Chitosan freeze gelled scaffold

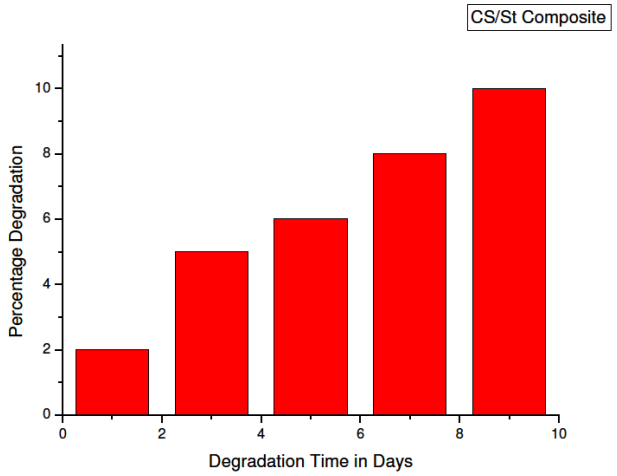


Fig.4.6.2 .Degradation study of Chitosan-Starch freeze gelled scaffold

5. CONCLUSIONS

- The scaffolds were successfully prepared by freeze gelation method within short time and less energy consumption.
- Both pure Chitosan and composite scaffolds showed interconnected pores with average pore size of 170 μm approximately when studied under SEM.
- XRD patterns of composite scaffold showed semi crystalline nature while pure Chitosan has amorphous nature.
- Both EDS and FTIR studies confirmed the presence of Chitosan and Starch in the requisite scaffolds.
- The prepared scaffold is studied to evaluate the degradation behavior and they were found to be suitable for tissue engineering applications.

The scaffold should be investigated for other characteristics like Mechanical and Swelling behavior. Their *in-vitro* biocompatibility (MTT Assay) also should be done before going for animal studies. This work ultimately will pave a way for economical scaffold manufacture for varied tissue engineering applications and help in establishing freeze gelation as the new scaffold manufacturing technique. At the same time studies done here will help in proving that composite Chitosan- Starch scaffolds are better than pure Chitosan ones.

6. REFERENCES

1. Langer R, Vacanti JP,Tissue engineering. Science, (1993), 920-926.
2. http://tissue.medicalengineer.co.uk/pages/tissueengineering/advantages-and-disadvantages-of-tissue-engineering.html
3. Khang, "A Manual for Biomaterials/Scaffold Fabrication Technology, Manuals in Biomedical Research ", vol-4, (2006), 222-226.
4. Joseph D. Bronzino, The Biomedical Engineering Handbook Third Edition Tissue Engineering and Artificial Organs, (2006), 582-599.
5. Antonios G. Micas, Formation of highly porous biodegradable scaffolds for tissue engineering, Electronic Journal of Biotechnology, Vol.3, (2006), 114-119.
6. Javier Nakamatsu, Processing and Characterization of Porous Structures from Chitosan and Starch for Tissue Engineering Scaffolds, Biomacromolecules, 7, (2006), 3345-3355.
7. Gabriela B.M. Ribeiro, Novel method to produce chitosan β-TCP scaffolds, Materials Letters 65, (2011), 275–277.
8. Jayachandran Venkatesan, Chitosan Composites for Bone Tissue Engineering—An Overview, *Mar*. Drugs, (2010), 2252-2266.
9. Uchida, Mixing conditions for cell scaffolds affect the bone formation induced by bone engineering with human bone marrow stromal cells, β-tricalcium phosphate granules, and rhBMP-2, Inc. J Biomed Mater Res 91A, (2006), 84–91.
10. Salgado, In vivo response to starch-based scaffolds designed for bone tissue engineering applications; Inc. J Biomed Mater Res 80A, (2007), 983–989.
11. Canonico S, The use of human fibrin glue in the surgical operations, Acta Biomed Ateneo Parmense, (2003), 21-25.
12. Kirilak, Fibrin sealant promotes migration and proliferation of human articular chondrocytes, possible involvement of thrombin and protease-activated receptors, International Journal of Molecular Medicine 17, (2006), 551-558.
13. Ming-Hua Ho , Porous polymer scaffolds surface-modified with arginine-glycine-aspartic acid enhance bone cell attachment and differentiation *in vitro,* Inc. J Biomed Mater Res 64A, (2003), 583–590.

14. Kambe, Effects of RGDS sequence genetically interfused in the silk fibroin light chain protein on chondrocyte adhesion and cartilage synthesis Biomaterials 31, (2011), 7503-7511.
15. Hsieh, Analysis of freeze-gelation and cross-linking processes for preparing porous chitosan scaffolds, Carbohydrate Polymers 67, (2007), 124–132.
16. Bhardwaj, Freeze-gelled silk fibroin protein scaffolds for potential applications in soft tissue engineering, International Journal of Biological Macromolecules 49, (2011), 260– 267.
17. Preparation of porous scaffolds by using freeze-extraction and freeze-gelation methods, Biomaterials 25, (2004), 129–138.
18. Hsieh, Analysis of freeze-gelation and cross-linking processes for preparing porous chitosan scaffolds, Carbohydrate Polymers 67, (2007), 124–132.
19. Orrego, Preparation and characterization of chitosan membranes by using a combined freeze gelation and mild crosslinking method, Bioprocess Biosyst Eng, (2009), 197–206.
20. K. Whang, C. H. Thomas and K. E. Healy, A novel method to fabricate bioabsorbable scaffolds Polymer, vol. 36, (1995).
21. Da-Ming Wang, Preparation of porous scaffolds by using freeze-extraction and freeze-gelation methods, Biomaterials 25, (2004).
22. Jiang Chang, Preparation and characterization of bioactive collagen/wollastonite composite scaffolds, Journal of Materials Science, Materials In Medicine, (2005).
23. Lein-Tuan Hou, Fabrication and release behavior of a novel freeze-gelled chitosan/ γ-PGA scaffold as a carrier for rhBMP-2, Dental Materials, (2006).
24. Hsyue-Jen Hsieh, Analysis of freeze-gelation and cross-linking processes for preparing porous chitosan scaffolds, Carbohydrate Polymers 67, (2007).
25. Hsyue-Jen Hsieh, Preparation and Cell Compatibility Evaluation of Chitosan/Collagen Composite Scaffolds Using Amino Acids as Crosslinking Bridges Published online in Wiley InterScience, (2007).
26. J.J. Cooper-White, Polyurethane/poly (lactic-co-glycolic) acid composite scaffolds fabricated by thermally induced phase separation, Biomaterials 28, (2007).
27. Principal IR Absorptions for Certain Functional Groups Interpretation of Infrared Spectra, A Practical Approach John Coates, Encyclopedia of Analytical Chemistry

6.APPENDIX

Typical Infrared Absorption Frequencies

	Stretching Vibrations			Bending Vibrations		
Functional Class	**Range (cm^{-1})**	**Intensity**	**Assignment**	**Range (cm^{-1})**	**Intensity**	**Assignment**
Alkanes	2850-3000	str	CH_3, CH_2 & CH 2 or 3 bands	1350-1470 1370-1390 720-725	med med wk	CH_2 & CH_3 deformation CH_3 deformation CH_2 rocking
Alkenes	3020-3100 1630-1680 1900-2000	med var str	=C-H & $=CH_2$ (usually sharp) C=C (symmetry reduces intensity) C=C asymmetric stretch	880-995 780-850 675-730	str med med	=C-H & $=CH_2$ (out-of-plane bending) cis-RCH=CHR
Alkynes	3300 2100-2250	str var	C-H (usually sharp) C≡C (symmetry reduces intensity)	600-700	str	C-H deformation
Arenes	3030 1600 & 1500	var med-wk	C-H (may be several bands) C=C (in ring) (2 bands) (3 if conjugated)	690-900	str-med	C-H bending & ring puckering
Alcohols & Phenols	3580-3650 3200-3550 970-1250	var str str	O-H (free), usually sharp O-H (H-bonded), usually broad C-O	1330-1430 650-770	med var-wk	O-H bending (in-plane) O-H bend (out-of-plane)
Amines	3400-3500 (dil. soln.) 3300-3400 (dil. soln.) 1000-1250	wk wk med	N-H (1°-amines), 2 bands N-H (2°-amines) C-N	1550-1650 660-900	med-str var	NH_2 scissoring (1°-amines) NH_2 & N-H wagging (shifts on H-bonding)
Aldehydes & Ketones	2690-2840(2 bands) 1720-1740 1710-1720 1690 1675 1745 1780	med str str str str str str	C-H (aldehyde C-H) C=O (saturated aldehyde) C=O (saturated ketone) aryl ketone α, β-unsaturation cyclopentanone cyclobutanone	1350-1360 1400-1450 1100	str str med	α-CH_3 bending α-CH_2 bending C-C-C bending
Carboxylic Acids & Derivatives	2500-3300 (acids) overlap C-H 1705-1720 (acids) 1210-1320 (acids) 1785-1815 (acyl halides) 1750 & 1820 (anhydrides) 1040-1100 1735-1750 (esters) 1000-1300 1630-1695(amides)	str str med-str str str str str str str	O-H (very broad) C=O (H-bonded) O-C (sometimes 2-peaks) C=O C=O (2-bands) O-C C=O O-C (2-bands) C=O (amide I band)	1395-1440 1590-1650 1500-1560	med med med	C-O-H bending N-H (1°-amide) II band N-H (2°-amide) II band
Nitriles	2240-2260	med	C≡N (sharp)			
Isocyanates,Isothiocyanates, Diimides, Azides & Ketenes	2100-2270	med	-N=C=O, -N=C=S -N=C=N-, $-N_3$, C=C=O			

Source http://www2.chemistry.msu.edu/faculty/reusch/VirtTxtJml/Spectrpy/InfraRed/infrared.htm